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Box Patent Application

Dear Sir:

Transmitted herewith for filing is the patent application of Barbara Papadopoulou et al. for **MACROPHAGE-INFECTING PARASITES EXPRESSING A GRANULOCYTE MACROPHAGE COLONY STIMULATING FACTOR.**

Enclosed are:

(X) 1. Specification with claims
(X) 2. Declaration and Power of Attorney - unsigned copy
(X) 3. 7 sheets of informal drawings

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TITLE OF INVENTION

MACROPHAGE-INFECTING PARASITES EXPRESSING A GRANULOCYTE MACROPHAGE COLONY STIMULATING FACTOR

FIELD OF INVENTION

The present invention relates to the field of molecular immunobiology and in particular to immunogenic preparations including vaccines comprising attenuated parasites.

REFERENCE TO RELATED APPLICATION

This application is a continuation-in-part of United States Patent Application No. 08/713,368 filed September 13, 1996.

BACKGROUND OF INVENTION

Parasite infection is responsible for a number of human and animal diseases.

Parasitic protozoa of the order Kinetoplastidae are the causative agents of several tropical diseases including sleeping sickness by *Trypanosoma brucei*, Chagas by *Trypanosoma cruzi*, visceral (kala-azar) and cutaneous (oriental sore) Leishmaniasis by *Leishmania donovani* and *Leishmania major* respectively. In particular *Leishmania* protozoans are the causative agents of human leishmaniasis, which includes a spectrum of diseases ranging from self-healing skin ulcers to fatal visceral infections. Human leishmaniasis is caused by at least thirteen different species and subspecies of parasites of the genus *Leishmania*. Leishmaniasis has been reported from about eighty countries and probably some 400,000 new cases occur each year. Recently the World Health Organization has reported 12 million people to be infected (ref. 1. Throughout this application various references are referred to in parenthesis to more fully describe the state of the art to which this invention pertains. Full bibliographic information for each citation is found at the end of the specification, immediately preceding the claims. The disclosure of these references are hereby

incorporated by reference into the present disclosure. A listing of the references appears at the end of the disclosure).

Untreated visceral leishmaniasis is usually fatal and mucocutaneous leishmaniasis produces mutilation by destruction of the naso-oropharyngeal cavity and, in some cases, death.

In addition a major health problem has been created in areas of high infection when blood is collected for transfusion purposes. Since blood is a carrier of the parasites, blood from an infected individual may be unknowingly transferred to a healthy individual.

The *Leishmania* protozoans exist as extracellular flagellated promastigotes in the alimentary tract of the sandfly in the free-living state, and are transmitted to the mammalian host through the bite of the insect vector. Once introduced, the promastigotes are taken up by macrophages, rapidly differentiate into non-flagellated amastigotes and start to multiply within the phagolysosomal compartment. As the infected cells rupture, amastigotes subsequently infect other macrophages giving rise to the various symptoms associated with leishmaniasis (refs. 1 and 2).

Leishmaniasis is, therefore, a serious disease and various types of vaccines against the disease have been developed, including live parasites; frozen promastigotes from culture; sonicated promastigotes; gamma-irradiated live promastigotes; and formalin-killed promastigotes treated with glucan (reviewed in, for example ref. 3). However, none of these approaches have provided efficacious vaccines.

Healing and progression of leishmaniasis are linked to the dissimilar expansion of functionally distinct CD4+ lymphocyte responses separated on the basis of their cytokine potential (ref. 4). T helper type 1

(Th1) subset produces interferon (IFN)-,γ and interleukin (IL)-2 and leads to resistance to *Leishmania* infection, whereas Th2 cells producing IL-4, IL-5 and IL-10 confer susceptibility (ref. 5). In mammalian hosts, *Leishmania* reside exclusively within mononuclear phagocytes, macrophages and monocytes. Cytokines can modulate macrophage differentiation by causing selective changes in macrophage gene expression, leading to alterations on macrophage functions (ref. 6). Macrophages, pre-incubated *in vitro* with cytokines prior to infection with *Leishmania*, acquire the capacity to kill the intracellular parasites (refs. 7 to 11). Furthermore, cytokines such as IFN-γ, TNF-α, IL-12 and GM-CSF have been used in anti-leishmanial therapy in experimental models (refs. 12 to 17). Expression of the IFN-γ gene has been performed in *L. major* trypanosomatids. When nude mice were infected with the IFN-γ expressing transfectant, the progression of the disease was considerably slower (ref. 17). The progression of the disease was not retarded in susceptible BALB/c mice however.

GM-CSF is a cytokine with multipontential hematopoietic function, stimulating the formation of granulocytes, macrophages, and eosinophils (ref. 18). It activates macrophage tumoricidal activity (ref. 19), increases macrophage killing of *Trypanosoma cruzi* (ref. 20) and enhances *in vitro* killing of *L. donovani* within macrophages (ref. 8).

Differences on the effect of this cytokine have been reported between experimental visceral and cutaneous infections (ref. 27). Although in the *L. donovani* model, GM-CSF demonstrates a clear-cut leishmanicidal activity *in vitro* and *in vivo* (refs. 28), the results obtained with *L. major* are conflicting suggesting that GM-CSF may play a positive (refs. 29,

10, 6), neutral (refs. 30, 4) or negative (ref. 31) host defense role.

Parasitic infection of macrophages and, in particular, *Leishmania* infection may lead to serious disease. It would be advantageous to provide attenuated strains of *Leishmania* and methods of production thereof, for use as antigens in immunogenic preparations, including vaccines, and the generation of diagnostic reagents.

SUMMARY OF THE INVENTION

The present invention is directed towards novel strains of *Leishmania* and other macrophage-infecting parasites, particularly attenuated strains. The novel macrophage-infecting parasites provided herein are useful for the preparation of immunogenic preparations including formulations for the treatment of hosts infected by *Leishmania* and vaccines against disease caused by infection by a virulent *Leishmania* strain and as tools for the generation of immunological and diagnostic reagents.

In accordance with one aspect of the present invention, there is provided a macrophage-infecting parasite expressing a granulocyte macrophage colony stimulating factor (GM-CSF) gene. The parasite may be a strain of *Leishmania*, including a strain of *Leishmania* selected from the group consisting of *Leishmania donovani*, *Leishmania braziliensis*, *Leishmania tarentolae*, *Leishmania major*, *Leishmania mexicana*, *Leishmania tropica* and *Leishmania aethiopica*.

The parasite may be one which is reduced in the ability of the strain to infect or survive in macrophages and hence is attenuated. Further, at least one gene of the parasite contributing to virulence thereof may be functionally disabled. Additionally, the parasite may be further modified to express at least one additional cytokine which may be macrophage-activating.

The GM-CSF may be of murine origin or human origin. Expression of the GM-CSF gene from the parasite may be achieved by providing a plasmid into which the GM-CSF gene is inserted downstream of a promoter. For *Leishmania*, the intergenic region of the α-tubulin gene of *L. enriettii* may be used and the GM-CSF gene may be inserted into a *Leishmania* expression vector, which may be a plasmid.

In a further aspect, the present invention provides an immunogenic composition comprising an attenuated form of the parasites as provided herein.

The immunogenic composition, for the parasite being a strain of *Leishmania* may be formulated for *in vivo* administration to a host, such as a primate, including humans, infected by *Leishmania* to treat such infection.

The immunogenic composition, for the parasite being a strain of *Leishmania*, may be formulated as a vaccine for *in vivo* administration to a host, such as a primate including humans, to confer protection against disease caused by a virulent strain of *Leishmania*, including *Leishmania donovani*, *Leishmania braziliensis*, *Leishmania tarentolae*, *Leishmania major*, *Leishmania mexicana*, *Leishmania tropica* and *Leishmania aethiopica*.

In an additional aspect, the invention provides a method of generating an immune response in a host, such as, a primate including humans, comprising administering thereto an immunoeffective amount of the immunogenic composition, as provided herein.

In yet an additional aspect, there is provided a method for producing a vaccine for protection against a disease caused by infection by a virulent strain of a macrophage-infecting parasite, including a virulent strain of *Leishmania*, including *Leishmania donovani*, *Leishmania braziliensis*, *Leishmania tarentolae*, *Leishmania mexicana*, *Leishmania tropica* and *Leishmania*

aethiopica, and comprising administering the immunogenic composition as provided herein to a test host to determine an amount and frequency of administration thereof to confer protection against disease caused by infection by the *Leishmania* parasite and formulating the immunogenic composition in a form suitable for administration to a treated host, including humans, in accordance with said determined amount and frequency of administration.

Advantages of the present invention include the provision of safe and attenuated strains of *Leishmania* and other macrophage-infecting parasites for the preparation of immunogenic compositions including vaccines and for the generation of immunological and diagnostic reagents.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the construction of *Leishmania* vectors expressing GM-CSF. Plasmids pneo-mGM CSF and pneo-hGM CSF were made by inserting the murine and human GM-CSF genes, respectively downstream of the intergenic region of the α-tubulin gene of *L. enriettii* into pSP72-αneoα *Leishmania* expression vector as described in the Examples below. The cross-hatch box corresponds to the α-tubulin gene intergenic region (ref. 21). S=*Sma*I, B=*Bnm*HI. Arrows indicate the orientation of transcription of the genes present in the expression vectors.

Figure 2 consists of two panels, A and B. Panel A is a southern blot of total genomic DNAs of *L. major* digested with *Bgl*II and hybridized to murine and human GM-CSF specific probes. Lane 1 corresponds to *L. major*-pneo and Lane 2 corresponds to *L. major* with either the murine or the human GM-CSF gene. Panel B shows mRNA expression in the *L. major*-GM CSF expressing cells.

Northern blot of total *Leishmania* RNAs hybridized to the same probes as with panel A above. Lanes are as in A.

Figure 3, comprising panels A and B, contains graphical representations of intramacrophage killing of *Leishmania donovani* amastigotes expressing the murine and human GM-CSF genes. *L. donovani* expressing either the murine or the human GM-CSF gene were harvested from stationary phase and counted with the Neubauer improved counting chamber. Murine macrophages from J774 cell line and human monocytes differentiated to macrophages (5×10^4 cells/well) were incubated with stationary phase *L. donovani* parasites (20:1, parasite-to-cell ratio) for 6 hours. After this initial incubation, free parasites were washed and fresh media was added to the wells and incubated for 48 and 72 hours. At these fixed time points cell cultures were dried and stained with Diff Quick in order to determine the level of infection. Panel A shows infection of murine macrophages. Panel B shows infection of human macrophages. The left graph of each panel corresponds to the percentage of infected macrophages and the right graph of each panel corresponds to the total number of amastigotes in 100 macrophage cells with time. ■ *L. donovani*-pneo; ● *L. donovani*-mGM CSF; ▲ *L. donovani*-hGM CSF.

Figure 4, comprising panels A and B, contains graphical representations of intracellular killing of *Leishmania major* amastigotes expressing the human GM-CSF gene. Human monocytes differentiated into macrophages were infected by *L. major* expressing the human GM-CSF gene as described in Fig. 3. Panel A shows the percentage of infected human macrophages with time. Panel B shows the total number of amastigotes in 100 macrophage cells with time. ■ *L. major*-pneo; ● *L.*

major-hGM CSF; ○ *L. major*-hGM CSF R* representing the reverse orientation of the GM-CSF gene.

Figure 5, comprising panels A and B, contains graphical representations of intracellular killing of *Leishmania major* amastigotes expressing the murine GM-CSF gene and reversion by an anti-GM CSF antibody. Murine macrophages were infected by *L. major* expressing the murine GM-CSF gene as described in Fig. 3. Panel A shows the percentage of infected murine macrophages with time. Panel B shows the total number of amastigotes in 100 macrophage cells with time. ■ *L. major*-pneo; ▲ *L. major*- mGM CSF; □ *L. major*-pneo infection with macrophages pre-treated with a concentration of an anti-GM CSF antibody capable to neutralize 50% of the GM-CSF activity; ○ *L. major*-mGM CSF infection with the same concentration of an anti-GMCSF antibody.

Figure 6 is a graphical representation of the inability of *L. major* expressing GM-CSF to promote infection with BALB/C susceptible mice. 2×10^2 *Leishmania* promastigotes expressing GM-CSF and also wild-type were injected into the footpad of eight mice per group. Infection was monitored by measuring the footpad lesion with a metric caliper over 6 weeks. ■ *L. major* m-GM CSF; ▲ *L. major* pSPYneosss mGM CSF.

Figure 7 is a graphical representation of the effect of GM-CSF *in vivo* $.1 \times 10^7$ stationary phase *L. Major* LV39 strain transfected either with pSPYneo (control) or with a mGM-CSF expression vector were used for infecting BALB/c mice. Cutaneous infection in mice was monitored by measuring the thickness in infected footpads with a metric caliper at weekly intervals. The net value for the footpad thickness (in mm) was

calculated by substracting the diameter of the uninfected footpad from the one of the infected footpad. The average and standard deviation of the results obtained from six mice per time point are shown. The data shown are representative of one experiment which has been repeated five times with essentially similar results. Δ = *L. Major - neo,* ▲ = *L. Major - mGM CSF.*

GENERAL DESCRIPTION OF THE INVENTION

Referring to Figure 1, there is shown vectors for expression of the Granulocyte Macrophage Colony Stimulating Factor (GM-CSF) in *Leishmania*.

To express GM-CSF in *Leishmania* the 800 bp intergenic region of the α-tubulin gene of *L. enriettii* was subcloned upstream of either the murine or human GM-CSF genes. The α-tubulin intergenic region provides the necessary signals for trans-splicing that are required for correct maturation of transcripts in *Leishmania* (ref. 21). The same sequences were used for the expression of the neomycin phosphotransferase gene (*neo*) as part of an α*neo*α cassette cloned in vector pSP72 (Fig. 1). These vectors were transfected by electroporation into *L. major* and *L. donovani* strains and transfectants resistant to G418 were selected on SDM-1% agar plates. Genomic DNAs were isolated from selected clones, digested with *Bgl*II to linearize the GM-CSF expression vectors and analyzed by Southern blot using GM-CSF specific probes either for the murine (m) or the human (h) genes.

5.8 kb and 6.2 kb fragments from the *L. major*-pneo-hGM CSF and pneo-mGM CSF transfectants, respectively were obtained as expected (Fig. 2A). Northern blot analysis of total *L. major* RNA showed that GM-CSF transcripts of the expected size for both the murine and the human genes were produced (Fig. 2B). Identical bands

were also obtained for the *L. donovani*-GM CSF transfectants.

The ability of *L. major*- and *L. donovani*-hGM CSF promastigotes to secrete GM-CSF was determined by direct ELISA assay. *Leishmania* cells were grown to stationary phase and the supernatants harvested after centrifugation. As assessed by ELISA, *Leishmania* expressing the hGM-CSF gene were associated with detectable amounts of GM-CSF protein in the media. The concentration of hGM-CSF detected in culture media ranged from 2.8 to 6.2 ng/ml. GM-CSF expressing parasites can be maintained in culture as promastigotes for several months without any obvious effect on growth or morphology. Therefore, GM-CSF expression is not detrimental to *Leishmania*.

Referring to Figure 3, there is shown the intramacrophage killing of *Leishmania donovani* expressing the murine and human GM-CSF genes. Phagocytes were infected with 10^6 stationary phase *Leishmania*-GM CSF and *Leishmania*-pneo control at a cell ratio of 1:20 for a period of 6 hours. The outcome of the infection *in vitro* was followed at 6, 24, 48, and 72 hours by microscopic examination. The intracellular survival of *Leishmania* amastigotes expressing GM-CSF significantly decreased inside both the murine and the human macrophages. More specifically, *L. donovani* amastigotes expressing either the murine or the human GM-CSF genes were eliminated 7 to 8-fold more rapidly inside macrophages than control cells 72 hours following infection (see Fig. 3A,B). Following the same infection period only 20% of macrophages were infected (Fig. 3A, B). Similar results were observed with *L. major* parasites expressing GM-CSF. Indeed, 72 hours following infection only 18-20% of macrophages were infected compared to 92-95% for the control strain (Figs. 4A and 5A). In addition, a 9-fold decrease was measured in the

total amastigote number found inside either human or murine macrophages infected with *L. major*-GM-CSF expressing parasites compared to those infected with the control-*neo* strain (Figs. 4B and 5B). The decrease in intracellular survival of *Leishmania* amastigotes expressing GM-CSF was a specific effect related to GM-CSF activity. Thus, murine macrophages were infected with *Leishmania* cells expressing either the human or the murine GM-CSF genes. Only parasites expressing the murine GM-CSF gene were eliminated inside macrophages (Fig. 3A). Furthermore, a construct in which the hGM-CSF gene was cloned in the reverse orientation in respect to the α-tubulin intergenic region necessary for expression in *Leishmania* was not capable of inducing parasite elimination in infected macrophages (Figure 4).

Finally, by pre-treating murine macrophages with an anti-mGM CSF polyclonal antibody prior to infection, the anti-leishmanial effect of GM-CSF was significantly decreased (Fig. 5). Indeed, an antibody concentration capable of neutralizing 50% of the GM-CSF activity blocked more than 50% of the intra-macrophage elimination of *L. major*-mGM CSF transfectants compared to the untreated cells (see Fig. 5). Similar results have been obtained using the *L. donovani* parasites.

Thus GM-CSF expression greatly reduces the viability of amastigotes inside murine or human macrophages. GM-CSF induce parasite killing by activating macrophages to enhance H_2O_2 release (refs. 18, 20) or most likely to produce more nitric oxide or to augment IFN-γ and IL-1 production (ref. 10). GM-CSF, as IFN-γ, can increase production of the anti-leishmanial cytokine TNF-α (refs. 12, 13, 32, 33, 15) and some of its biological effects might be amplified through the release of this cytokine.

Referring to Figure 6, there is shown the inability of *L. major* expressing GM-CSF to promote infection of BALB/c susceptible mice. Two series of animals were inoculated into the footpad with either 2 x 10^7 *L. major*-mGM CSF recombinant parasites (a clone) or with *L. major*-pSPYneo control strain. Eight mice were used for each group. The outcome of the infection of these mice was followed by weekly measurement of the footpad lesions. As shown in Fig. 6, after six weeks post-infection, a significant decrease in the development of cutaneous lesion was observed in animals inoculated with the GM-CSF expressing parasites compared to those infected with the neo control strain.

Figure 7 is representative data of a series of experiments in which BALB/c mice were infected with *L. major* expressing either the GM-CSF gene or the neo gene as a control. The procedure is the same as that for Figure 6, except that groups of six mice were inoculated with 1 x 10^7 *L. major* mGM CSF recombinant parasites or with *L. major* as pSPYneo control strain. The net value for footpad thickness was calculated by substracting the diameter of the uninfected footpad from one of the infected footpad. The mice infected with *Leishmania* experessing mGM-CSF were followed for 11 weeks. The average and standard derviation obtained from the six mice per time point are shown in Figure 7.

The mice infected with *Leishmania* expressing GM-CSF showed no inflammation or lesions at the level of the footpad for the first 4 to 5 weeks post-infection compared to the control mice where lesions were very large after the same time period and the mice needed to be sacrified. From the fifth week, inflammation at the level of the footpads infected with GM-CSF expressing parasites started to be visable and slowly increased with time over the three-months of observation. The

majority of the mice did not develop important lesions such as those seen with the control parasites.

The *Leishmania*-GM-CSF expressing parasites provided herein provide means to create favorable immune responses in a host. Parasites expressing cytokines provide effective vaccines for leishmaniasis by mediating their own destruction while establishing protective Th1-mediated immunity.

EXAMPLES

The above disclosure generally describes the present invention. A more complete understanding can be obtained by reference to the following specific Examples. These Examples are described solely for purposes of illustration and are not intended to limit the scope of the invention. Changes in form and substitution of equivalents are contemplated as circumstances may suggest or render expedient. Although specific terms have been employed herein, such terms are intended in a descriptive sense and not for purposes of limitations.

Methods of molecular genetics, protein biochemistry and immunology used but not explicitly described in the disclosure and these Examples are amply reported in the scientific literature and are well within the ability of those skilled in the art.

Example 1

This Example describes techniques of recombinant DNA.

Leishmania vector pneo-mGM CSF expressing the murine-GM-CSF gene was made as follows. A 1.2 kb *Kpn*I-*Kpn*I fragment isolated from plasmid pXMT2 containing the mGM-CSF gene and flanking sequences was filled in with T4 DNA polymerase (New England Biolabs) to create blunt ends and subcloned into the *Bam*HI site of vector pSP72-αneoα filled in with Klenow DNA polymerase (New England

Biolabs) to yield pneo-mGM CSF (Figure 1). Vector pSP72-αneoα was generated by subcloning the 2.6 kb *Bam*HI-*Sma*I *neo*-cassette from pGEM3-neo (ref. 23) containing the *neo* gene flanked by the intergenic regions of the α-tubulin gene of *L. enriettii* (ref. 21) into the *Bam*HI-*Sma*I of pSP72 (Promega). The *Leishmania* construct to express the human GM-CSF gene was made by subcloning an 800 bp *Xho*I-*Xho*I fragment from vector pXMT1 containing the hGM-CSF gene and flanking sequences into the *Sal*I site of pSP72-αneoα to yield pneo-hGM CSF (Figure 1). Construct pneo-hGM CSF R* (Figure 1) corresponds to the reverse orientation of the human GM-CSF gene with respect to the αneoα cassette in such way that specific gene expression cannot occur.

Total genomic DNA from *Leishmania* was prepared as described (ref. 26), digested with *Bgl*II resolved on 0.7% agarose gels and transferred to nylon membranes (Hybond-N, Amersham). Total RNAs from *L. major*-pneo and GM-CSF transfectants were prepared using Trizol (Gibco BRL). Southern and Northern blots, hybridizations and washings were done following standard procedures and the results are shown in Figure 2. The GM-CSF probes used correspond to a 1.2 kb *Kpn*I-*Kpn*I fragment for the mGM-CSF gene and a 800 bp *Xho*I-*Xho*I fragment for the hGM-CSF gene.

Example 2

This Example describes the culturing and transfection of *Leishmania*.

Leishmania major MHOM/IL/67/JERICHO II; and *Leishmania donovani* MHOM/IN/80/DD8 are WRAIR/WHO reference strains obtained from the ATCC. All strains were grown in SDM-79 medium (ref. 22) supplemented with 10% fetal bovine serum (FBS) (Multicell, Wisent Inc.) and 5 µg/ml of hemin. Approximately 15 µg of pneo-GM CSF vector DNA was used to transfect *L. donovani* and *L.*

major cells by electroporation as described (refs. 23, 24). Transfectants were selected with 40 µg/ml of G-418 (Geneticin, Gibco-BRL). *Leishmania*-GM CSF and control *Leishmania*-pneo transfectants were seeded at low concentration (3 x 10^4 cells per culture dish) in 5 ml of SDM-79 medium. After 72 hours the cell density was obtained by measuring the absorbance at 600 nm using an automated microplate reader (Reader 510 from Organon Tecknika Inc., Austria). Murine macrophage cell line J774, obtained from ATCC, was cultured in Dulbecco's modified Eagle's medium (D-MEM, Gibco-BRL) supplemented with 10% FBS. Human peripheral blood monocytes were isolated from heparinized venous blood of normal donors by the Canadian Red Cross. Cells were centrifuged over Ficoll-Paque gradient (Pharmacia) as previously described (ref. 25). After several washes, cells were resuspended in RPMI 1640 medium (Gibco-BRL) containing 10% of human serum (Gibco-BRL). In order to differentiate monocytes into macrophages, 3 x 10^6 peripheral blood leukocytes were adhered and cultured for 5 days at 37°C in 5% CO_2/95% air in a humidified atmosphere.

Example 3

This Example describes *in vitro* macrophage infection.

The capacity of *Leishmania*-GM CSF transfectants to infect murine and human macrophages *in vitro* was tested in comparison to control *Leishmania*-pneo as follows. Murine and human macrophages were seeded (200 µl per well, 5 x 10^4 cells/ml) into 8 wells chamber slides, and were infected with *L. major*-pneo and *L. major*-hGM CSF at a parasite to cell ratio of 20:1 for a period of 6 hours. The experiment was repeated with *L. donovani*-pneo and *L. donovani*-hGM CSF and -mGM CSF. Following this incubation, non-engulfed parasites were removed by 3 to

5 washes with warm medium and chambers were replenished with 500 µl of fresh culture medium. The level of infection was determined at 6, 24, 48, and 72 hours by optical microscopy examination following Diff Quick staining of cell preparations. The results obtained are shown in Figures 3 and 4 for *L. donovani* and *L. major* respectively. The results show that the GM CSF expression greatly reduces the viability of amastigotes inside murine or human macrophages.

Example 4

This Example describes the neutralization of GM-CSF activity.

Two hundred µl of J774 murine macrophages were incubated for 1 hour at 37°C in the presence of 1 µg/ml of an anti-murine GM-CSF polyclonal antibody (R&D Systems) prior to infection with *Leishmania* cells. Pre-incubated macrophages were then infected with *L major*-mGM CSF and wild-type parasites as described above in Example 3. The results obtained are shown in Figure 5. The anti-Leishmanial effect of GM-CSF was formed to be significantly decreased by the antibody treatment.

Example 5

This Example describes ELISA immunoassays.

Supernatants from *L. major*- and *L. donovani*-hGM CSF-containing parasites and from controls carrying only the *neo* vector were harvested by centrifugation following 5 days of culture to stationary phase with a reached density of 2×10^7 promastigotes/ml. Supernatants were assayed directly using a mAb ELISA kit (R&D Systems) as recommended by the manufacturers. Standard curves for quantification and comparison were generated using a recombinant hGM-CSF protein. *Leishmania* expressing the hGM-CSF gene were associated with detectable amounts of GM-CSF protein at the media, ranging from 2.8 to 6.2 ng/ml.

SUMMARY OF THE DISCLOSURE

In summary of the present disclosure, there is provided a macrophage-infecting parasite expressing a granulocyte macrophage stimulating factor (GM-CSF) gene for use as a vaccine against or treatment of a parasitic infection. In particular, the parasite may be a strain of *Leishmania*. Modifications are possible within the scope of the invention.

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CLAIMS

What we claim is:

1. A macrophage infecting parasite expressing a granulocyte macrophage colony stimulating factor (GM-CSF) gene.
2. The parasite of claim 1 which is a strain of *Leishmania*.
3. The parasite of claim 2 wherein said strain of *Leishmania* is selected from the group consisting of *Leishmania donovani, Leishmania braziliensis, Leishmania tarentolae, Leishmania major, Leishmania mexicana, Leishmania tropica* and *Leishmania aethiopica*.
4. The parasite of claim 3 which is reduced in the ability of said strain to infect or survive within macrophages.
5. The parasite of claim 2 wherein said GM-CSF gene is of murine origin.
6. The parasite of claim 2 wherein said GM-CSF gene is of human origin.
7. The parasite of claim 2 wherein said GM-CSF gene is expressed using the α-tubulin intergenic sequences of *Leishmania enrietti*.
8. The parasite of claim 7 wherein said GM-CSF gene is expressed from a plasmid.
9. The parasite of claim 1 wherein at least one gene of the parasite contributing to virulence thereof has been functionally disabled.
10. The parasite of claim 1 which expresses at least one additional cytokine.
11. An immunogenic composition comprising an attenuated form of the parasite of claim 1.
12. The immunogenic composition of claim 11 wherein the parasite is a strain of *Leishmania* and the composition is formulated for *in vivo* administration to a host infected by *Leishmania* to treat said infection.

13. The immunogenic composition of claim 11 wherein said parasite is a strain of *Leishmania* and said composition is formulated as a vaccine for *in vivo* administration to a host to confer protection against disease caused by a virulent strain of *Leishmania*.

14. The immunogenic composition of claim 13 wherein the virulent strain is selected from the group consisting of *Leishmania donovani, Leishmania braziliensis, Leishmania tarentolae, Leishmania major, Leishmania mexicana, Leishmania tropica* and *Leishmania aethiopica*.

15. The immunogenic composition of claim 12 or 13 wherein the host is a primate.

16. The immunogenic composition of claim 12 or 13 wherein the host is a human.

17. A method of generating an immune response in a host comprising administering thereto an immunoeffective amount of the immunogenic composition of claim 11.

18. A method for producing a vaccine for protection against a disease caused by infection by a virulent strain of a macrophage-infecting parasite, comprising:

administering the immunogenic composition of claim 11 to a test host to determine an amount and frequency of administration thereof to confer protection against the disease; and

formulating the immunogenic composition in a form suitable for administration to a treated host in accordance with said determined amount and frequency of administration.

19. The method of claim 18 wherein said parasite is a strain of *Leishmania*.

20. The method of claim 19 wherein the treated host is a human.

ABSTRACT OF THE DISCLOSURE

Strains of *Leishmania* and other macrophage-infecting parasites are provided which express the GM-CSF gene which are useful in treating hosts infected by the parasite and in protecting hosts against disease caused by infection of hosts by parasites. The parasites are reduced in their ability to infect or survive in macrophages and hence are attenuated. At least one gene of the parasite contributing to the virulence thereto may be functionally disabled. The attenuated strains may be used for administration to a host (a) to treat a host infected by *Leishmania* or (b) to confer protection against disease caused by a virulent *Leishmania* strain, or as a diagnostic reagent.

Combined Declaration and Power of Attorney for United States Patent Application

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name;

I believe I am the original, first and joint inventor of the subject matter which is claimed and for which a patent is sought on the invention entitled: **MACROPHAGE-INFECTING PARASITES EXPRESSING A GRANULOCYTE MACROPHAGE COLONY STIMULATING FACTOR**, the specification of which is attached hereto.

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, S.1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code, S.119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Prior Foreign Application(s)			Priority Claimed	
			Yes	No
(Number)	(Country)	(Day/Month/Year Filed)		

I hereby claim the benefit under Title 35, United States Code, S.120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, S.112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, S.1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

08/713,768	13-Sept-96	Pending
(Appln. Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that wilful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such wilful false statements may jeopardize the validity of the application or any patent issued thereon.

I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith:

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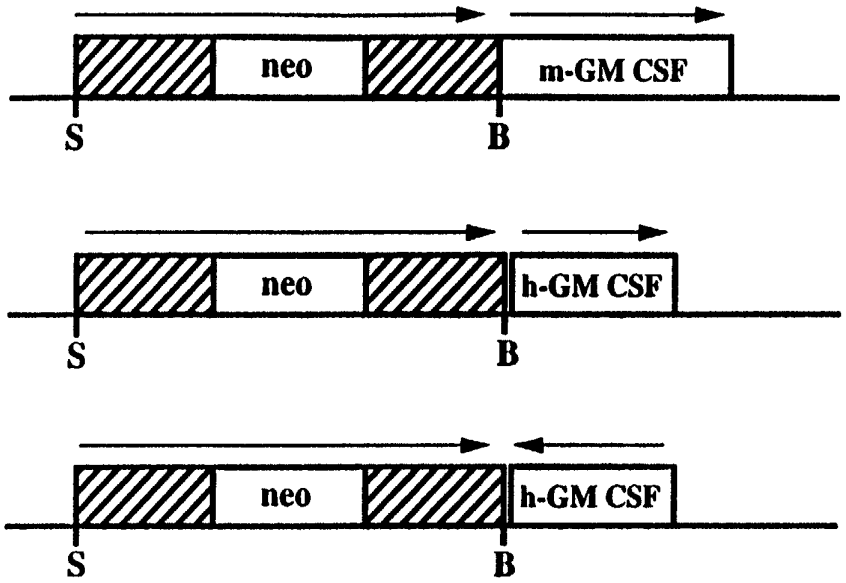


pneo-mGM CSF

pneo-hGM CSF

pneo-hGM CSF-R*

Fig. 1

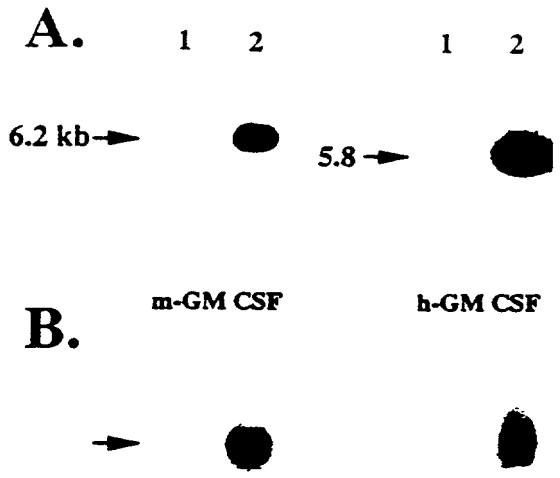
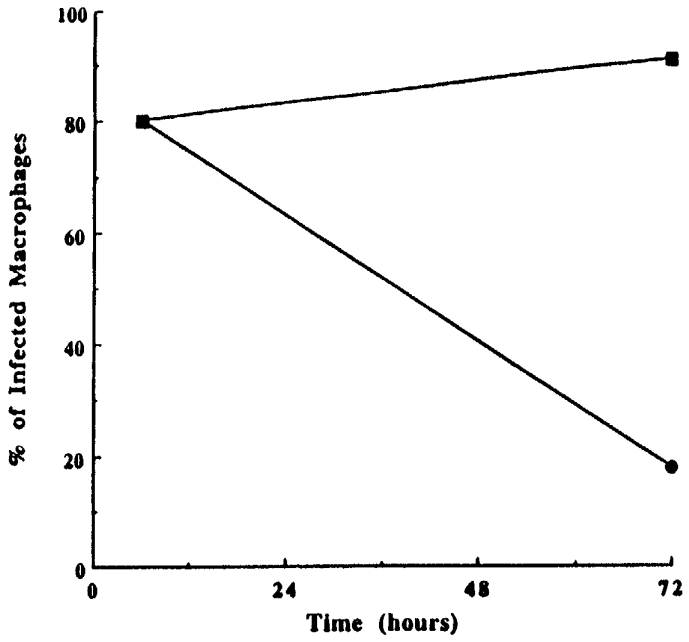
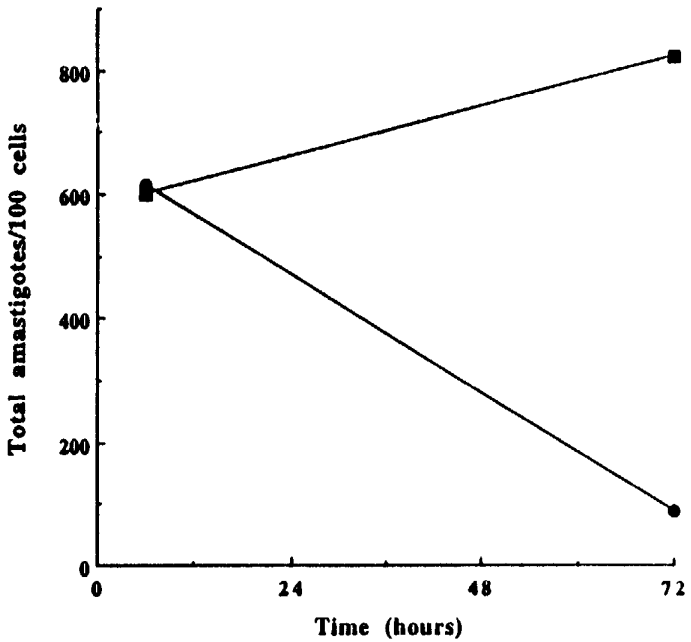


FIG. 2

A.





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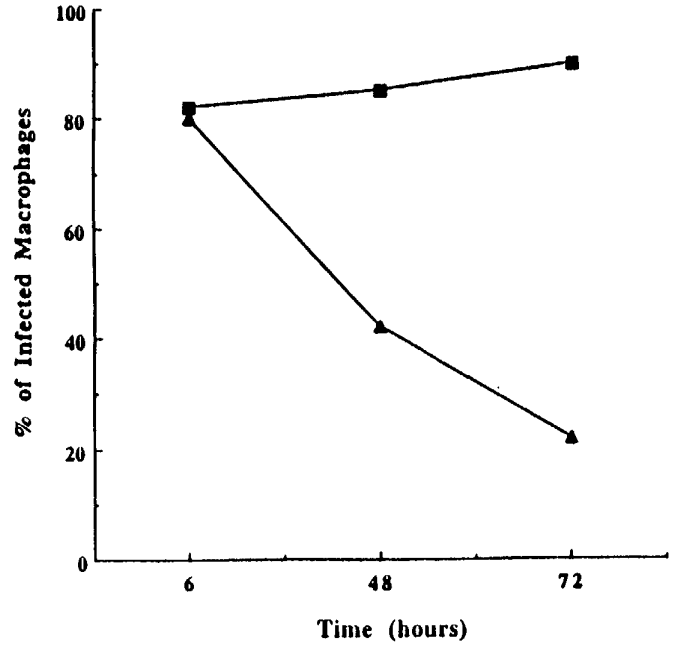


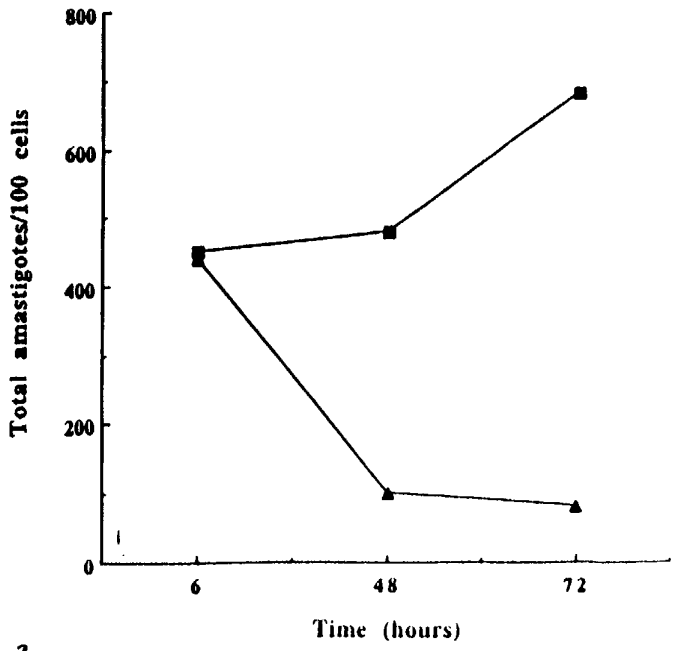


Fig. 1

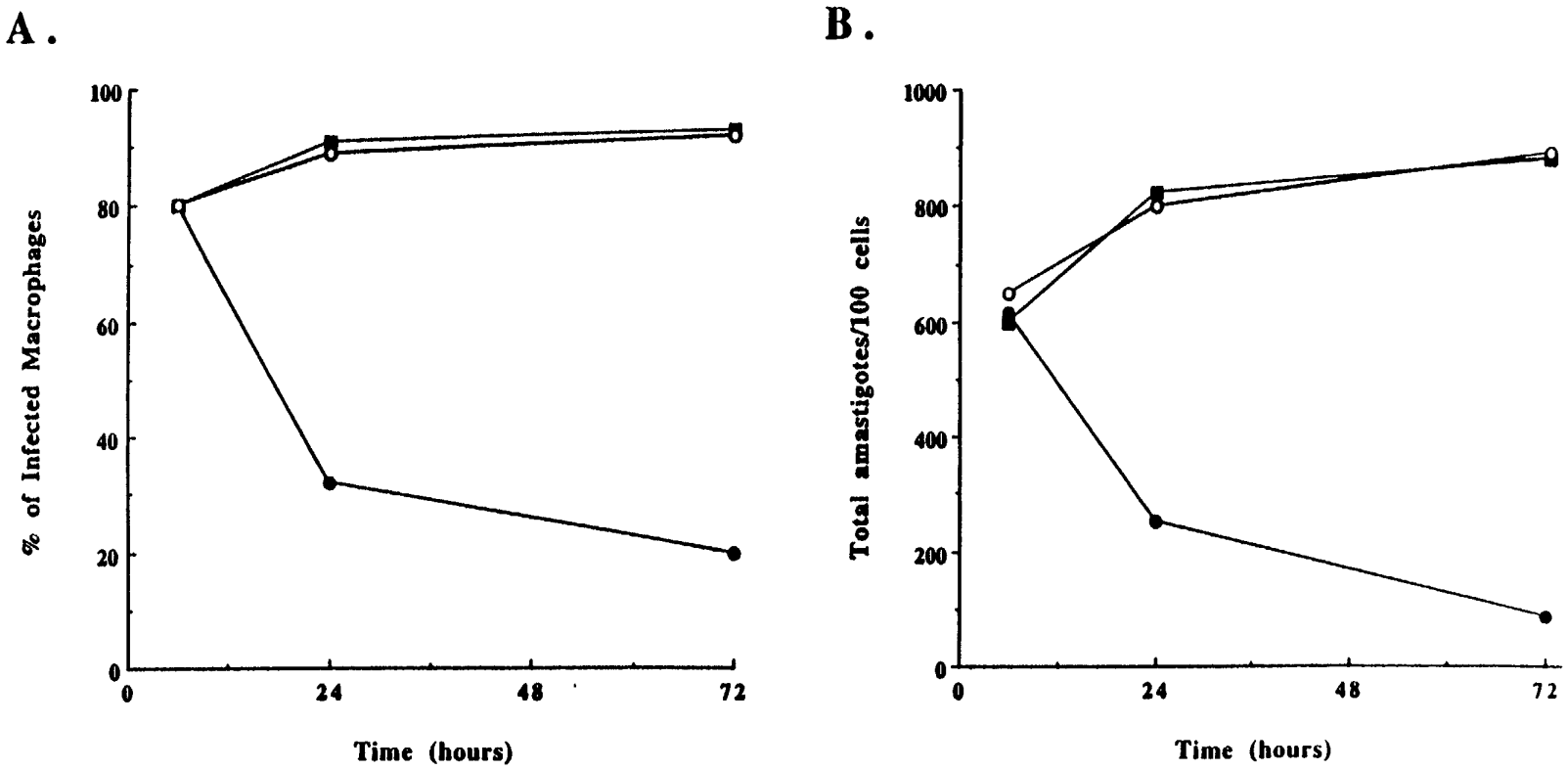
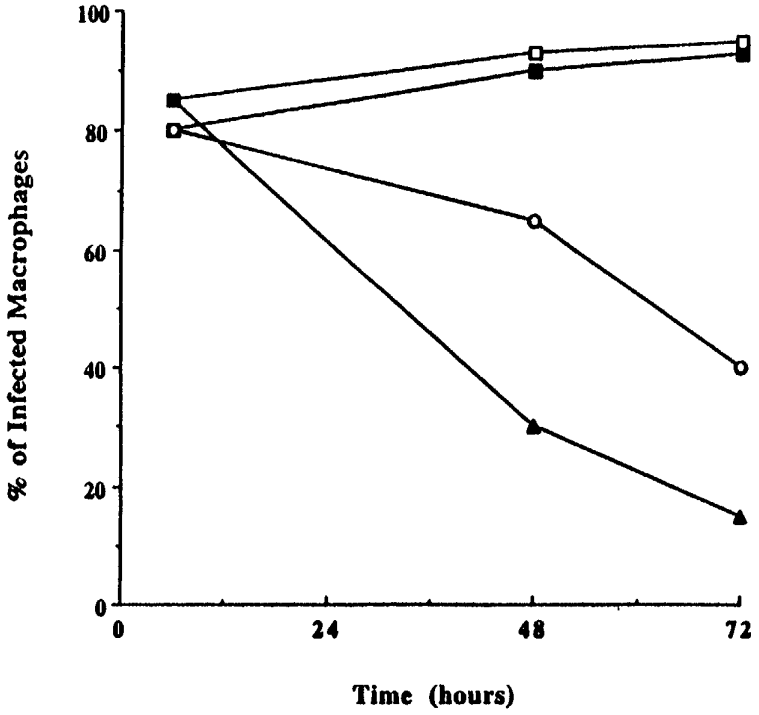

A.
% of Infected Macrophages
100
80
60
40
20
0
0
24
48
72
Time (hours)
B.
Total amastigotes/100 cells
1000
800
600
400
200
0
0
24
48
72
Time (hours)

Fig. 4

A.



B.

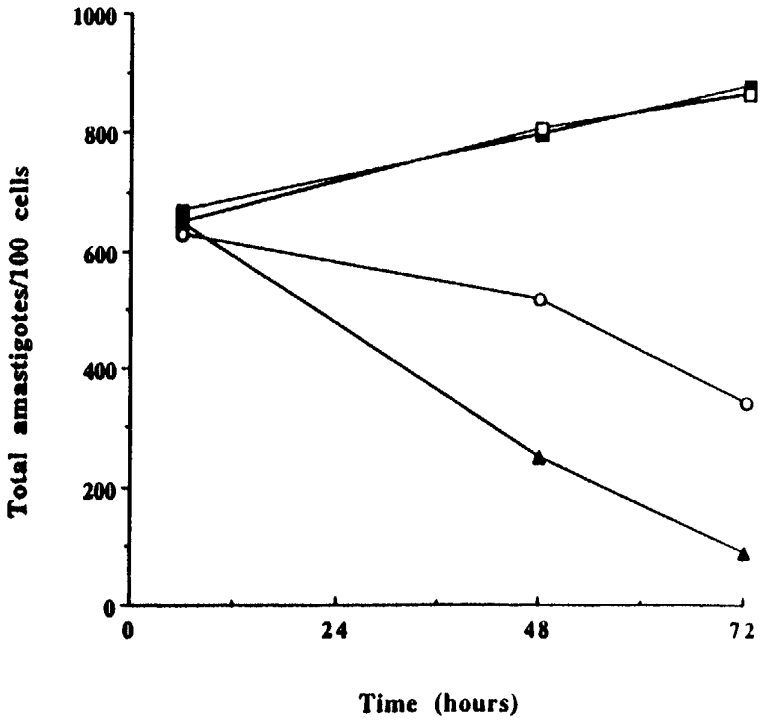


Fig 5

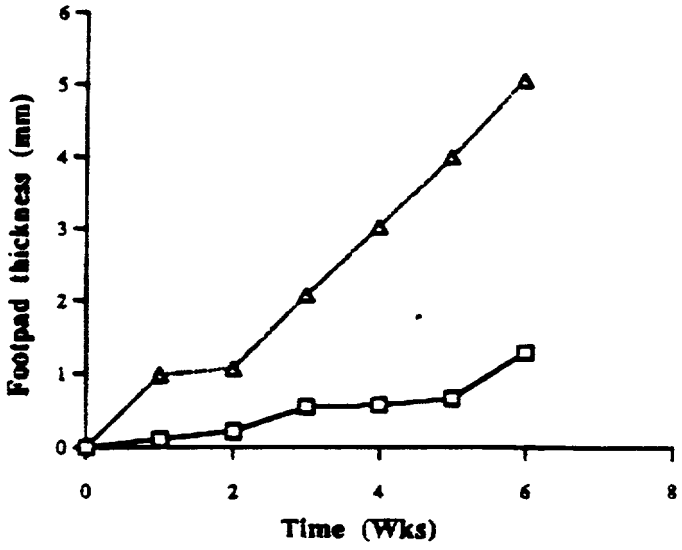


Fig. 6. Inability of *L. major* expressing GM-CSF to promote infection into BALB/c susceptible mice. $2X10^7$ *Leishmania* promastigotes expressing GM-CSF and also wild-type were injected into footpad of 8 mice per group. Infection was monitored by measuring the footpad lesion with a metric caliper over 6 weeks.
❐ *L major m-GM CSF*, Δ *L.major pSPYneosss*

Fig 7